



Thomas Jefferson University  
**Jefferson Digital Commons**

Department of Emergency Medicine Faculty Papers

Department of Emergency Medicine

5-6-2015

# Adiponectin attenuates lung fibroblasts activation and pulmonary fibrosis induced by paraquat.

Rong Yao

*Emergency Medicine Department, West China Hospital, Sichuan University*

Yu Cao

*Emergency Medicine Department, West China Hospital, Sichuan University*

Ya-Rong He

*Emergency Medicine Department, West China Hospital, Sichuan University*

Wayne Bond Lau

*Department of Emergency Medicine, Thomas Jefferson University, 1015 Walnut Street, Philadelphia, PA, 19107, USA,  
waynebond.lau@jefferson.edu*

Zhi Zeng

*Emergency Medicine Department, West China Hospital, Sichuan University*

*See next page for additional authors*

[Let us know how access to this document benefits you](#)

Follow this and additional works at: <http://jdc.jefferson.edu/emfp>

 Part of the [Emergency Medicine Commons](#)

## Recommended Citation

Yao, Rong; Cao, Yu; He, Ya-Rong; Lau, Wayne Bond; Zeng, Zhi; and Liang, Zong-An, "Adiponectin attenuates lung fibroblasts activation and pulmonary fibrosis induced by paraquat." (2015).

*Department of Emergency Medicine Faculty Papers. Paper 38.*

<http://jdc.jefferson.edu/emfp/38>

This Article is brought to you for free and open access by the Jefferson Digital Commons. The Jefferson Digital Commons is a service of Thomas Jefferson University's [Center for Teaching and Learning \(CTL\)](#). The Commons is a showcase for Jefferson books and journals, peer-reviewed scholarly publications, unique historical collections from the University archives, and teaching tools. The Jefferson Digital Commons allows researchers and interested readers anywhere in the world to learn about and keep up to date with Jefferson scholarship. This article has been accepted for inclusion in Department of Emergency Medicine Faculty Papers by an authorized administrator of the Jefferson Digital Commons. For more information, please contact: [JeffersonDigitalCommons@jefferson.edu](mailto:JeffersonDigitalCommons@jefferson.edu).

---

**Authors**

Rong Yao, Yu Cao, Ya-Rong He, Wayne Bond Lau, Zhi Zeng, and Zong-An Liang

RESEARCH ARTICLE

# Adiponectin Attenuates Lung Fibroblasts Activation and Pulmonary Fibrosis Induced by Paraquat

Rong Yao<sup>1☯</sup>, Yu Cao<sup>1☯</sup>, Ya-rong He<sup>1</sup>, Wayne Bond Lau<sup>2</sup>, Zhi Zeng<sup>1‡\*</sup>, Zong-an Liang<sup>3‡\*</sup>

**1** Emergency Medicine Department, West China Hospital, Sichuan University, 37 Guoxue Road, Chengdu, Sichuan, 610041, PR China, **2** Emergency Medicine Department of Thomas Jefferson University Hospital, 1025 Walnut Street, 808 College Building, Philadelphia, PA, 19107, United States of America, **3** Respiratory Department, West China Hospital, Sichuan University, 37 Guoxue Road, Chengdu, Sichuan, 610041, PR China

☯ These authors contributed equally to this work.

‡ These authors also contributed equally to this work.

\* [1362960346@qq.com](mailto:1362960346@qq.com) (ZL); [530291451@qq.com](mailto:530291451@qq.com) (ZZ)



## OPEN ACCESS

**Citation:** Yao R, Cao Y, He Y-r, Lau WB, Zeng Z, Liang Z-a (2015) Adiponectin Attenuates Lung Fibroblasts Activation and Pulmonary Fibrosis Induced by Paraquat. PLoS ONE 10(5): e0125169. doi:10.1371/journal.pone.0125169

**Academic Editor:** Qiang Ding, University of Alabama at Birmingham, UNITED STATES

**Received:** October 8, 2014

**Accepted:** March 21, 2015

**Published:** May 6, 2015

**Copyright:** © 2015 Yao et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper.

**Funding:** This work was supported by the National Natural Science Foundation of China (No. 30900493 and No. 81471836) and the Science and Technology Department Foundation of Sichuan Province (No. 2013JY0011).

**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

Pulmonary fibrosis is one of the most common complications of paraquat (PQ) poisoning, which demands for more effective therapies. Accumulating evidence suggests adiponectin (APN) may be a promising therapy against fibrotic diseases. In the current study, we determine whether the exogenous globular APN isoform protects against pulmonary fibrosis in PQ-treated mice and human lung fibroblasts, and dissect the responsible underlying mechanisms. BALB/C mice were divided into control group, PQ group, PQ + low-dose APN group, and PQ + high-dose APN group. Mice were sacrificed 3, 7, 14, and 21 days after PQ treatment. We compared pulmonary histopathological changes among different groups on the basis of fibrosis scores, TGF- $\beta_1$ , CTGF and  $\alpha$ -SMA pulmonary content via Western blot and real-time quantitative fluorescence-PCR (RT-PCR). Blood levels of MMP-9 and TIMP-1 were determined by ELISA. Human lung fibroblasts WI-38 were divided into control group, PQ group, APN group, and APN receptor (AdipoR) 1 small-interfering RNA (siRNA) group. Fibroblasts were collected 24, 48, and 72 hours after PQ exposure for assay. Cell viability and apoptosis were determined via Kit-8 (CCK-8) and fluorescein Annexin V-FITC/PI double labeling. The protein and mRNA expression level of collagen type III, AdipoR1, and AdipoR2 were measured by Western blot and RT-PCR. APN treatment significantly decreased the lung fibrosis scores, protein and mRNA expression of pulmonary TGF- $\beta_1$ , CTGF and  $\alpha$ -SMA content, and blood MMP-9 and TIMP-1 in a dose-dependent manner ( $p < 0.05$ ). Pre-treatment with APN significantly attenuated the reduced cell viability and up-regulated collagen type III expression induced by PQ in lung fibroblasts, ( $p < 0.05$ ). APN pretreatment up-regulated AdipoR1, but not AdipoR2, expression in WI-38 fibroblasts. AdipoR1 siRNA abrogated APN-mediated protective effects in PQ-exposed fibroblasts. Taken together, our data suggests APN protects against PQ-induced pulmonary fibrosis in a dose-dependent manner, via suppression of lung fibroblast activation. Functional AdipoR1 are expressed by

human WI-38 lung fibroblasts, suggesting potential future clinical applicability of APN against pulmonary fibrosis.

## Introduction

Paraquat (PQ) is a fast acting, effective herbicide used worldwide. Human exposure to PQ results in pulmonary accumulation of PQ, causing acute injury and ultimately fibrosis [1, 2]. Despite significant progress made in understanding the molecular mechanisms responsible for PQ-related lung fibrosis, the prognosis remains poor, due in part to lack of effective therapies [3, 4].

Adiponectin (APN), an adipocytokine predominantly secreted by adipocytes and found at high levels in plasma, has received great attention in the past decade for its anti-insulin resistance, anti-atherosclerotic and anti-inflammatory effects [5, 6, 7]. Several observations suggest to the potential role of APN against various fibrotic diseases. APN-knockout mice exhibit augmented carbon tetrachloride-induced liver fibrosis, while adenoviral-mediated increased APN expression prevented liver fibrosis in wild-type mice [8]. In a subtotal renal ablation model of APN-knockout mice, observed exacerbation of albuminuria and renal fibrosis was attenuated by adenoviral-mediated APN overexpression [9]. APN exerts protective effect against angiotensin II-induced cardiac fibrosis [10]. While evidence suggesting the anti-fibrotic effect of APN has accumulated, the effect of APN upon fibrotic processes in pulmonary tissue remains incompletely understood. Whether APN may attenuate PQ-induced lung fibrosis has never been previously investigated.

Therefore, the objectives of the current study are: 1) to determine whether APN exerts protective effect against PQ-induced pulmonary fibrosis; 2) to determine the effect of APN upon the expression of fibrosis-related cytokines and lung fibroblast activation; 3) to determine whether adiponectin receptors (AdipoR) are expressed by human lung fibroblasts; and if so, 4) if pulmonary fibroblast AdipoR are functional, and play a role in APN-mediated effects upon PQ-induced pulmonary fibrosis.

## Materials and Methods

This study was carried out in strict accordance with the ethical standards in the 1986 Directive 86/609/EEC, "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" and the "Guiding Principles in the Use of Animals in Toxicology", adopted by the Society of Toxicology in 1989. This protocol was approved by the Committee on the Ethics of Animal Experiments at Sichuan University.

## Materials

PQ dichloride (1, 1'-dimethyl-4, 4'-bipyridinium dichloride) was purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Recombinant mouse globular APN isoform was from Adipobio (Beijing, China). Antibodies against transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), connective tissue growth factor (CTGF),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and collagen type III were all from Abcam (Boston MA, US). The enzyme linked immunosorbent assay (ELISA) kits of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were from USCN Life Science Inc (Wuhan, China) and Cusabio Life Science (Wuhan, China), respectively.

## Animals and treatment

Male BALB/c mice (Dossy Biological Technology Co. Ltd, Chengdu, China) were housed in a temperature ( $22\pm 2^{\circ}\text{C}$ ) and humidity-controlled room with free access to fresh water and standard laboratory food. After 1 week of conditioning in a 12 hour light/dark cycle, 80 mice were randomly divided into four groups: 1) control group (saline injection), 2) PQ group (10 mg/kg delivered intraperitoneally, IP), 3) PQ + low-dose APN group (APN, 100  $\mu\text{g}/\text{kg}$  by tail vein injection at 0, 24, and 48 hours after PQ exposure), 4) PQ + high-dose APN group (APN, 500  $\mu\text{g}/\text{kg}$  by tail vein injection at 0, 24, and 48 hours after PQ exposure). Doses for high/low APN concentration were determined in preliminary experiments (data not shown). All reasonable efforts were made to ameliorate suffering, including anesthesia using pentobarbital (50 mg/kg, IP) for isolation of affected mice. Mice were monitored daily for signs of pain or distress. Moribund mice were humanely sacrificed as described below. Study design and biometric planning of each experiment was performed in accordance with a biostatistician. Mice were sacrificed for sample preparation by cervical dislocation after anesthesia. For each experiment, the single animal was an experimental unit. Serum/pulmonary samples were harvested at 3, 7, 14, and 21 days after PQ injection.

## Histopathological assessment of lung tissue

Lung tissue, fixed in 10% neutral-buffered formalin, was blocked in paraffin by an automated processor, and subjected to graded alcohol, xylene, and paraffin treatments. Sections of 4  $\mu\text{m}$  thickness were stained by hematoxylin and eosin (H&E) or Masson trichrome stain to assess lung injury/fibrosis extent. The severity of lung fibrosis was graded on a scale of 0 (normal lung) to 8 (most severe fibrotic lung), as previously reported by Ashcroft and colleagues [11]. At least 10 high-power fields (400 x) were evaluated per lung sample, scored independently by two investigators in blinded fashion.

## Cell culture and grouping

Human lung fibroblasts WI-38 obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies), 100 UI/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (both from Cellgro, USA), and were incubated in a humidified incubator at  $37^{\circ}\text{C}$  in a 95% air/5%  $\text{CO}_2$  atmosphere until reaching confluence. From the 4th day onward, the culture medium was removed and replaced with fresh medium every 72 hours. Confluent cells were subsequently harvested for passage by a solution of trypsin (0.25%) and EDTA (0.02%) (Roche, Swiss).

When cells reached 80% confluence, they were randomly divided into four groups: Control group, PQ group, APN group, and AdipoR1 siRNA group. Lung fibroblasts WI-38 were incubated with 0.3 mmol/L PQ for 24 hours, with and without pretreatment with APN (5  $\mu\text{g}/\text{mL}$ ) for 2 hours. The AdipoR1 siRNA group cultured lung fibroblasts WI-38 were transfected with AdipoR1 siRNA via lipofectamine (Invitrogen) per manufacturer's instructions, and then treated as per the APN group. Un-stimulated lung fibroblasts served as controls. Cells were collected for assays 24, 48, and 72 hours after PQ stimulation.

## Cell viability and apoptosis assay

Cell viability and apoptosis were measured by Kit-8 (CCK-8) (Beyotime, China) and fluorescence Annexin V-FITC/PI double labeling (Boehringer Mannheim GmbH), respectively, per manufacturer protocol.

For CCK-8 assay, growing cell density was adjusted to  $5 \times 10^4$  cells/ml. Cells were placed in 96-well dishes, incubated for 24 hours at 37°C in a 95% air/5% CO<sub>2</sub> atmosphere, serum-starved for 24 hours in DMEM, transfected with reagents, and continuously incubated for 24 hours under routine conditions. After discarding supernatant, 10 µl CCK-8 solution was added to each well. Cell culture continued for 1 hour. The OD of cells in the wells was quantitated at 450 nm via enzyme-linked immunometric meter (Thermo, USA).

For Annexin V-FITC/PI staining and flow cytometry, pulmonary fibroblasts were collected, washed with PBS, and adjusted to density  $1 \times 10^6$ /ml. Cells were centrifuged at 500 rpm for 5 minutes at room temperature, and the supernatant was discarded. Cells were washed with PBS and stained with Annexin V/PI detection kit (Boehringer Mannheim GmbH) per manufacturer's instructions. Both 5 µl Annexin V-FITC and 10 µl PI were added to 100 µl of suspended cells, and incubated for 15 minutes at room temperature in the dark. The samples were analyzed by FACS (Beckman Coulter Equipment) via Cell Quest Research Software (Becton Dickinson). To detect the same cell with two different lasers, the beams were separated from each other by a 40 ms time delay. A 15 mW argon ion laser emitting a 488 nm beam was the excitation source for Annexin V-FITC (FL1) and PI (FL2). Green fluorescence of Annexin V was collected with a 515 nm band pass filter; the red fluorescence of PI was collected with a 610 nm band pass filter.

### siRNA transfection

RNA interference was used to down-regulate the expression of AdipoR1 in lung fibroblasts. The siRNA were synthesized by Bioneer (Shanghai). The siRNA sequences used for targeted silencing of human AdipoR1 were 5'-ATCAATGTATCTTAAGGCGTGAATTCTCTAG-3'.

For gene knockdown experiments, lung fibroblasts WI-38 were plated in 6-cm dishes and cultured for 48 hours in DMEM containing 10% FBS. After incubating 24 hours in medium without FBS and antibiotics, cells were transfected with AdipoR1 siRNA via Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. After 48 additional hours of culture, cells were recultured in DMEM containing 10% FBS.

### Western blot

Western blot analysis was employed to detect the protein expression level of TGF-β<sub>1</sub>, CTGF, and α-SMA in lung tissue, and collagen type III, α-SMA, and AdipoR1/R2 in human lung WI-38 fibroblasts. Whole lung tissue homogenate or WI-38 lung fibroblasts lysates were prepared via lysis buffer [TianGen Biotech (Beijing) Co., Ltd.]. The lung tissue homogenate or cell lysates were separated by electrophoresis on 10% SDS-PAGE. Gels were transferred to a nitrocellulose membrane, blocked in 5% nonfat dry milk diluted in Tris-buffered saline. Blots were incubated overnight with anti-TGF-β<sub>1</sub>, anti-CTGF, anti-α-SMA, anti-collagen type III, anti-AdipoR1, or anti-AdipoR2 antibodies (all from Abcam), at 4°C. Washed blots were incubated at room temperature for 60 minutes with a secondary biotinylated antibody (Zhongshan Golden Bridge Bio-technology, Beijing). After three 10 minute washings with TBST, membranes were analyzed by Western (Bio-Rad Laboratories Inc., Hercules, CA). Sample loadings were normalized by Western blot analysis with anti-GAPDH (Abcam).

### RT-PCR

We quantified mRNA expression in lung tissue by real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from lung samples or lung fibroblasts via RNA Prep Pure [TianGen Biotech (Beijing) Co., Ltd.], and reverse transcribed into cDNA per manufacturer's instructions. The quantity and quality of isolated RNAs were evaluated via absorbance

**Table 1. Primer sequences used for RT-PCR.**

Gene	forward primer (5'–3')	reverse primer (5'–3')
TGFβ <sub>1</sub>	5'–AGGAGACGGAATACAGGGCT–3'	5'–CCACGTAGTAGACGATGGGC–3'
CTGF	5'–AGCGGTGAGTCCTTCCAAAG–3'	5'–TTCATGATCTCGCCATCGGG–3'
α-SMA	5'–GACAATGGCTCTGGGCTCTGTAA–3'	5'–ATGCCATGTTCTATCGGGTACTT–3'
Col III	5'–TGGTCCCCAAGGTGTCAAAG–3'	5'–GGGGGTCTGGGTACCATTAA–3'
AdipoR1	5'–GCGATAACGAGGTGGAGTGT–3'	5'–GATGGGTCCAGATGTTGCCA–3'
AdipoR2	5'–ATAGGGCAGATAGGCTGGTTGA–3'	5'–GGATCCGGGCAGCATACA–3'

doi:10.1371/journal.pone.0125169.t001

measurements at 260 and 280 nm wavelengths. The mRNA expression level of TGF-β<sub>1</sub>, CTGF, and α-SMA in lung tissue, and α-SMA, collagen type III, and AdipoR1/R2 in human lung WI-38 fibroblasts were measured via RT-PCR by an ICycler IQ Real time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA). Primers designed are listed in [Table 1](#).

## Statistical analysis

All results are presented as mean±SEM. Comparisons between groups at each time point were made by one-way analysis of variance (ANOVA), followed by Student Newman—Keuls test. P values less than 0.05 were considered statistically significant.

## Results

### APN attenuates PQ-induced pulmonary fibrosis in dose-dependent manner

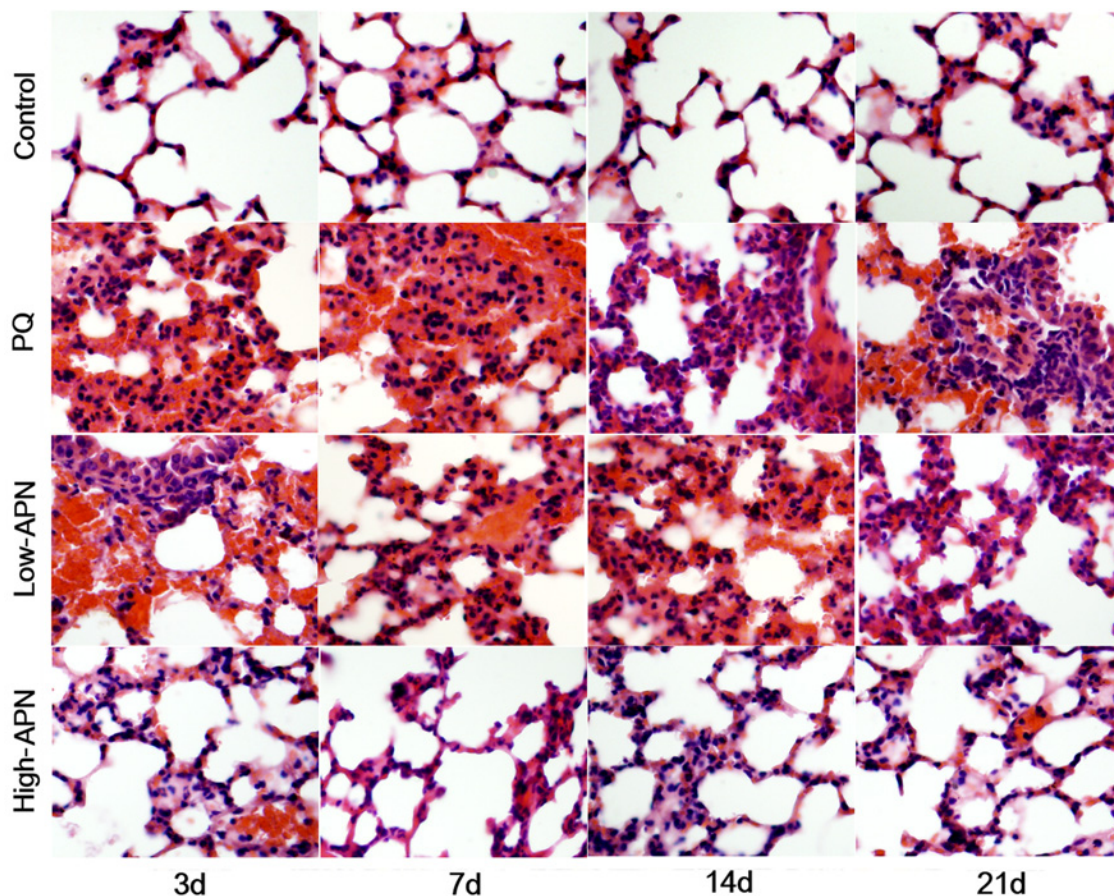
Histologic tissue changes mediated by PQ and APN were evaluated by H&E or Masson trichrome staining. Compared to control ([Fig 1](#)), PQ exposure induced a marked inflammatory response characterized by interstitial edema and widespread inflammatory cell (lymphocytes and histiocytes) infiltration in the alveolar space and septum, substantially thickening and degrading normal alveolar structure by day 3. Masson trichrome stain revealed markedly increased collagen deposition, predominately in the thickened alveolar regions and small bronchioles ([Fig 2](#)), beginning day 3, and peaking day 21. APN treatment attenuated the marked interstitial thickening, inflammatory responses, and collagen accumulation induced by PQ in a dose-dependent manner.

Lung sections were randomized and scored blindly, assessed for interstitial thickening of alveolar or bronchiolar walls, collagen deposition, and damaged lung architecture. PQ exposure increased the lung fibrosis score assigned to histologic appearance ([Table 2](#)). APN treatment attenuated the lung fibrosis score in dose-dependent manner, particularly between days 14–21 ( $P<0.05$ ).

### APN decreases PQ-induced TGF-β<sub>1</sub>, CTGF and α-SMA expression in lung tissue

To further investigate the specific anti-fibrotic effect of APN, the expression pattern of fibrosis-related cytokines in mouse lung tissue was evaluated by Western blot and RT-PCR. TGF-β<sub>1</sub> has been demonstrated to be the most potent and direct stimulator of fibroblast proliferation and collagen production, and is requisite for pulmonary fibrosis of various etiologies [12]. We investigated whether the TGF-β<sub>1</sub> signaling pathway is involved in the anti-fibrosis effect of APN upon PQ-induced lung fibrosis. As shown in [Fig 3A](#), PQ exposure progressively increased





**Fig 1. H&E stained histological evaluation of mouse lungs in the control, PQ, PQ + low-dose APN, and PQ + high-dose APN groups on days 3, 7, 14, 21 after PQ injection (x200 magnification).** Compared with control group, PQ (10 mg/kg) intoxication showed progressively interstitial edema and inflammatory cell infiltration in the alveolar space and septum from day 3, reaching a peak on day 14. Both low high-dose (100  $\mu$ g/kg) and high-dose (500  $\mu$ g/kg) APN groups showed remarkably decreased interstitial edema and inflammatory cell infiltration, in a dose-dependent manner.

doi:10.1371/journal.pone.0125169.g001

TGF- $\beta_1$  expression in lung tissue, while APN treatment decreased lung TGF- $\beta_1$  protein and mRNA levels significantly at every measured time point ( $P < 0.05$ ) in dose-dependent fashion.

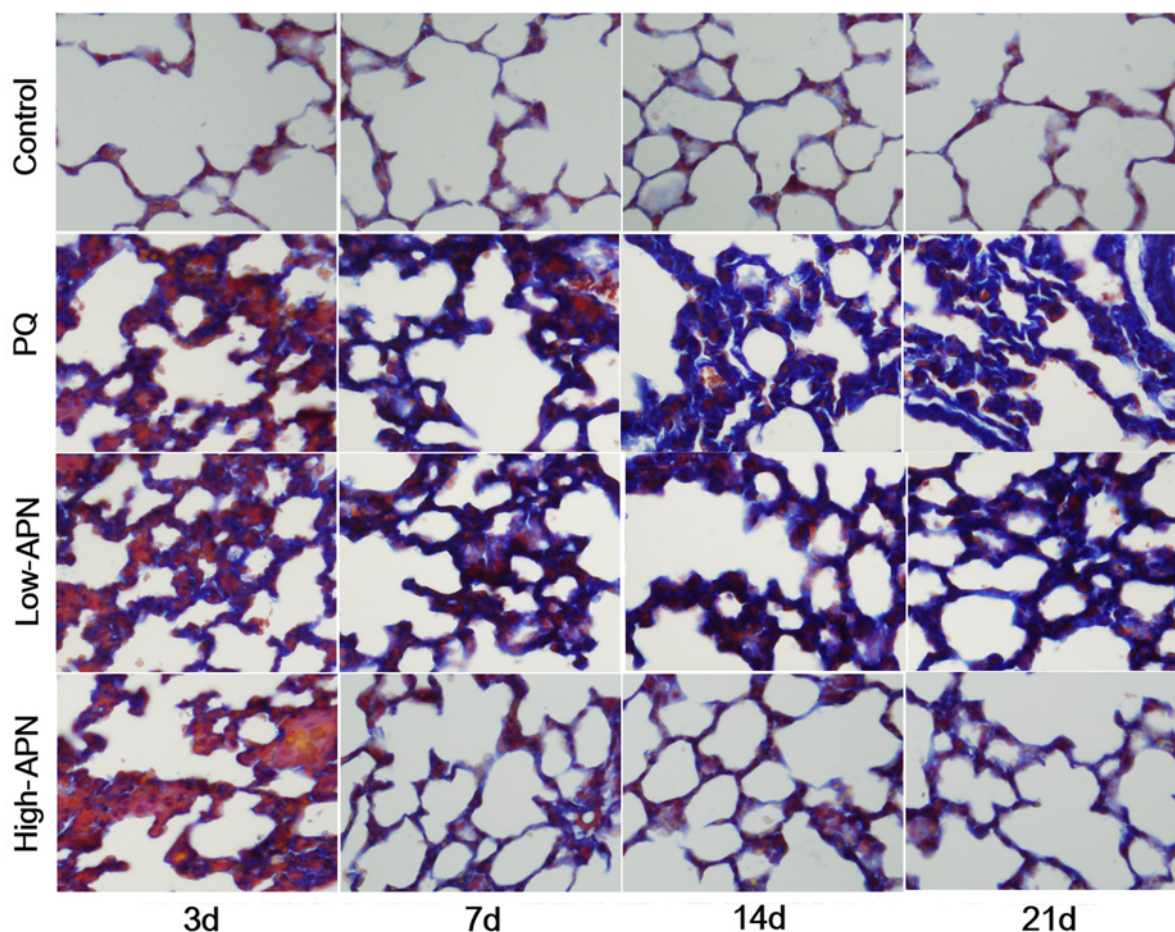
CTGF is considered both a downstream and cooperative mediator of TGF- $\beta_1$ , inducing fibroblasts to become myofibroblasts [13]. We analyzed the effect of APN upon pulmonary CTGF expression in mice subjected to PQ. PQ increased lung CTGF expression, which was significantly inhibited by both low and high-dose APN ( $P < 0.05$ , Fig 3B).

TGF- $\beta_1$  promotes myofibroblast differentiation and induces the expression of  $\alpha$ -SMA in lung fibroblasts [12]. Increased expression of  $\alpha$ -SMA is evidence of increased extracellular matrix (ECM)-producing myofibroblasts, an important feature of active fibrotic disease. We demonstrated PQ increased  $\alpha$ -SMA expression in mouse lung tissue, and APN significantly inhibited PQ-mediated augmented  $\alpha$ -SMA expression ( $P < 0.05$ , Fig 3C). Together, these results indicate PQ stimulated myofibroblast transformation, a process blocked by APN.

### APN decreases PQ-induced elevation of MMP-9 and TIMP-1 in circulation

Of the matrix metalloproteinase members, MMP-9 is implicated in lung fibrosis [14]. TIMP-1, a physiological inhibitor of MMP-9, is upregulated during fibrosis [15, 16]. We determined the





**Fig 2. Histological evaluation of mice lungs in the control, PQ, PQ+ low-dose APN or PQ+ high-dose APN group on day 3, 7, 14, 21 after PQ injection with Masson stain ( $\times 200$  magnification).** Compared to control, PQ (10 mg/kg) exposure increased fibrosis (seen as blue collagen deposition) in the alveolar regions and small bronchioles from day 3, peaking on day 21. Both low-dose (100  $\mu$ g/kg) and high-dose (500  $\mu$ g/kg) APN groups exhibited decreased collagen deposition, in a dose-dependent manner.

doi:10.1371/journal.pone.0125169.g002

effect of APN upon expression/activity of both MMP-9 and TIMP-1. PQ exposure significantly increased MMP-9 and TIMP-1 blood levels, peaking on day 3 and 7 respectively. APN treatment significantly reduced this increase from day 3 after initial PQ exposure in a dose-dependent manner (Figs 4 and 5). It is noteworthy that both MMP-9 and TIMP-1 levels peaked on day 7 after APN treatment, suggesting a correction of imbalanced MMP-9/TIMP-1 ratio induced by PQ.

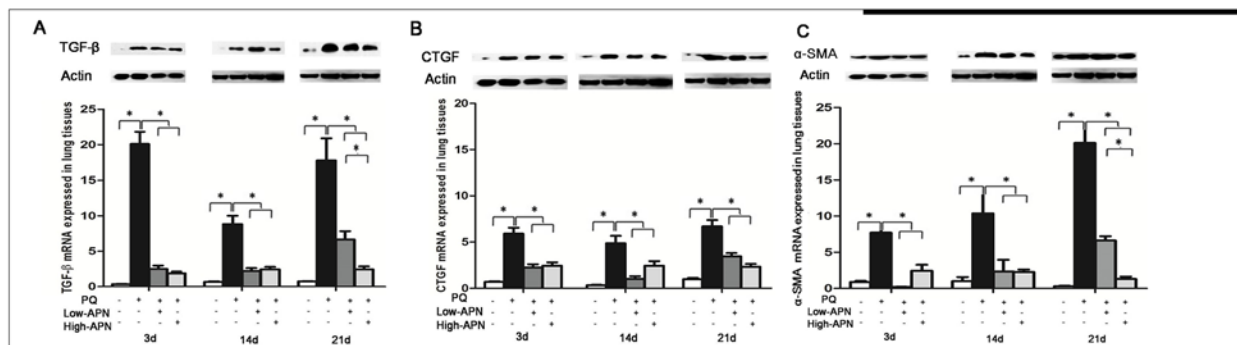
**Table 2. Lung fibrosis scores across groups.**

	Day 3	Day 7	Day 14	Day 21
control group	0	0	0	0
PQ group	3(2–5)	5(3–7)	7(5–8)*	8*
PQ + low-dose APN group	3(2–5)	4(2–5)	5(3–7)	6(4–7)*
PQ + high-dose APN group	2(1–4)	3(2–5)	3(2–5) <sup>#</sup>	3(2–5) <sup>#</sup>

\*: PQ group VS control group,  $p < 0.05$

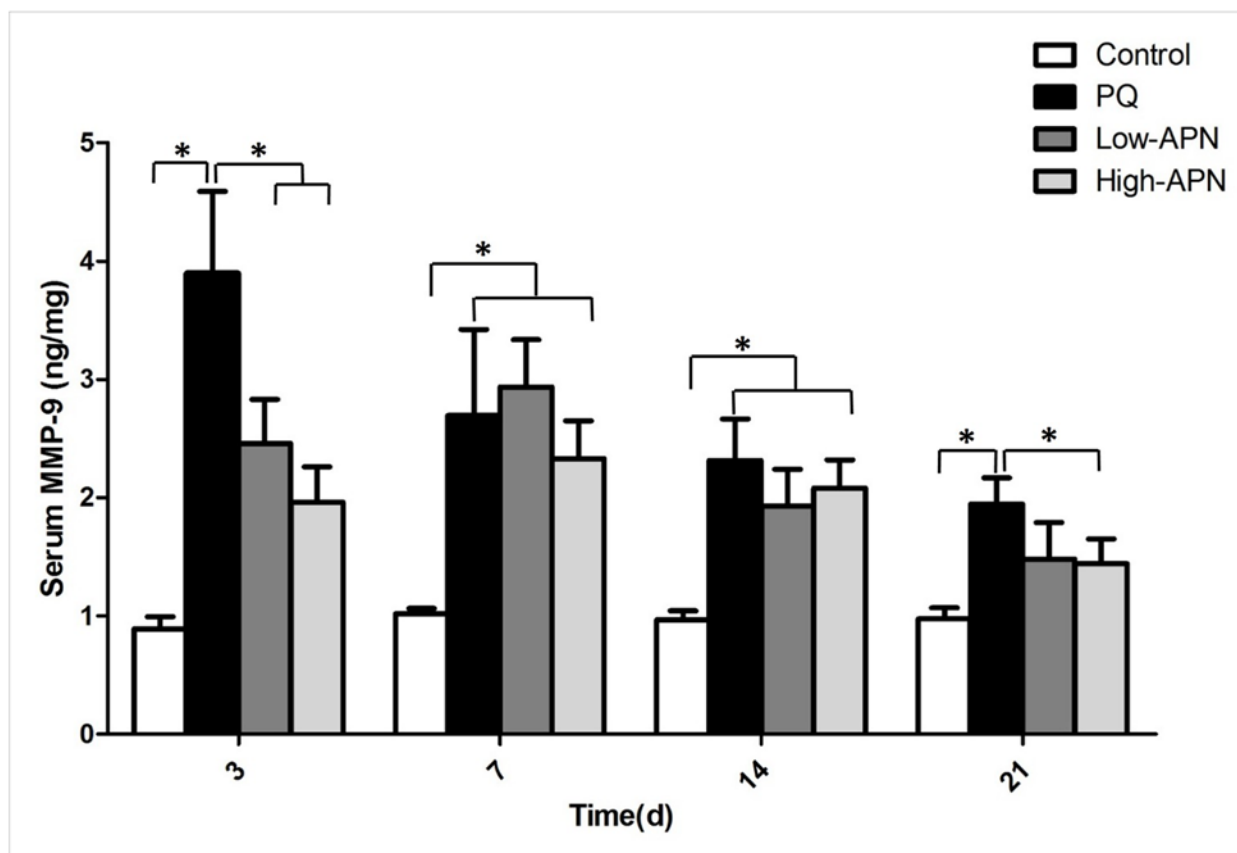
<sup>#</sup>: PQ + APN group VS PQ group,  $p < 0.05$

doi:10.1371/journal.pone.0125169.t002



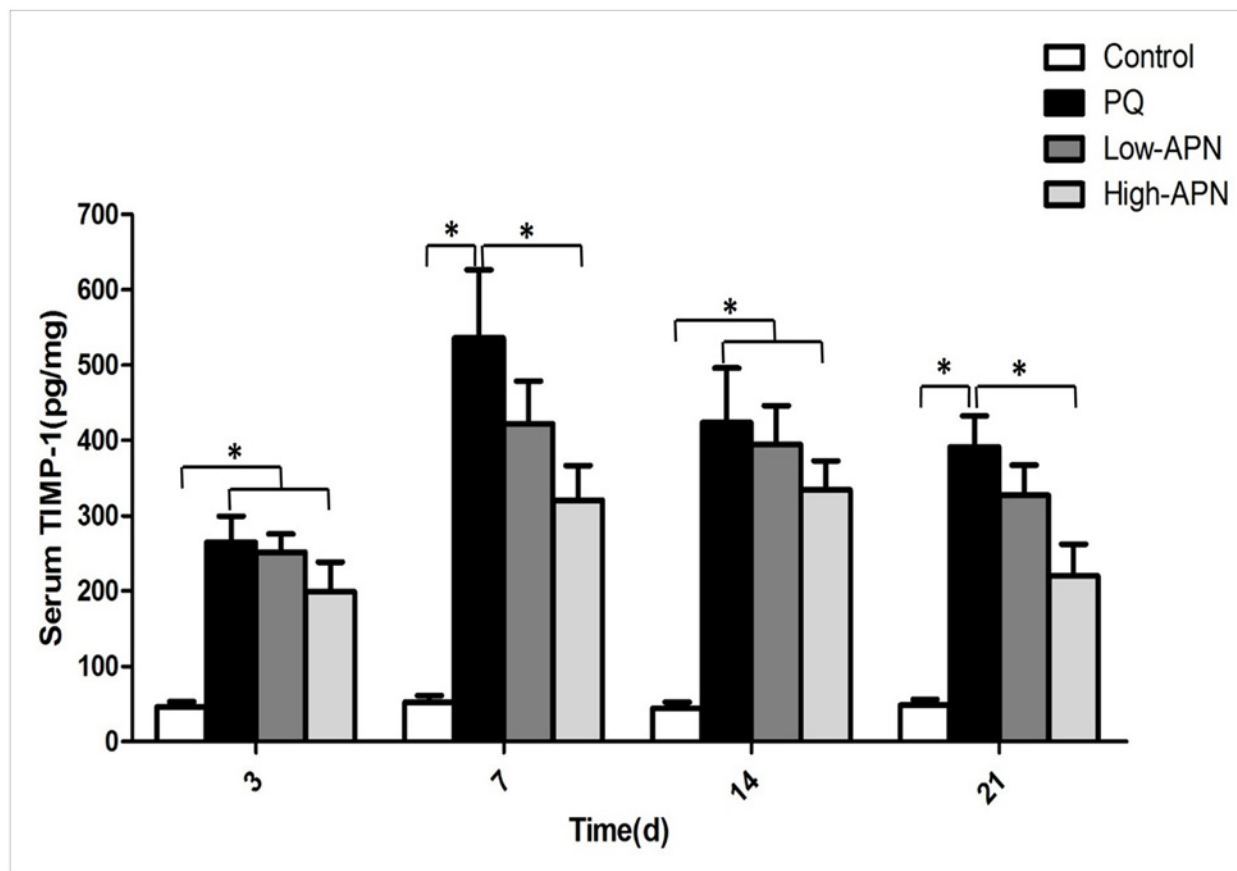
**Fig 3. Effect of APN on TGF- $\beta$ 1, CTGF and  $\alpha$ -SMA expression in lung tissues from control animals or PQ-exposed animals, treated with vehicle or APN.** PQ (10 mg/kg, IP) increased, whereas APN (100  $\mu$ g/kg or 500 $\mu$ g/kg, IV) reversed, TGF- $\beta$ 1 (A), CTGF (B) and  $\alpha$ -SMA (C) expression in mice lung tissues at 3, 14 and 21 d after PQ injection. The protein and mRNA expression were determined via Western blot and RT-PCR, respectively. Values represent the mean  $\pm$  standard error of the mean of four parallel measurements. \* $P < 0.05$ . APN, adiponectin; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; CTGF, connective tissue growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; PQ, paraquat.

doi:10.1371/journal.pone.0125169.g003



**Fig 4. Effect of APN on MMP-9 blood levels in control animals or PQ-exposed animals, treated with vehicle or APN.** PQ (10 mg/kg, IP) increased, whereas APN (100  $\mu$ g/kg or 500 $\mu$ g/kg, IV) reversed, MMP-9 levels at 3, 7, 14 and 21 d after PQ injection. MMP-9 levels were determined by an enzyme-linked immunosorbent assay. Values represent the mean  $\pm$  standard error of the mean of four parallel measurements. \* $P < 0.05$ . APN, adiponectin; MMP-9, matrix metalloproteinase-9; PQ, paraquat.

doi:10.1371/journal.pone.0125169.g004



**Fig 5. Effect of APN on TIMP-1 blood levels in control animals or PQ-exposed animals, treated with vehicle or APN.** PQ (10 mg/kg, IP) increased, whereas APN (100 µg/kg or 500µg/kg, IV) reversed, TIMP-1 levels at 3, 7, 14 and 21 d after PQ injection. TIMP-1 levels were determined by an enzyme-linked immunosorbent assay. Values represent the mean ± standard error of the mean of four parallel measurements. \*P<0.05. APN, adiponectin; TIMP-1, tissue inhibitor of metalloproteinase-1; PQ, paraquat.

doi:10.1371/journal.pone.0125169.g005

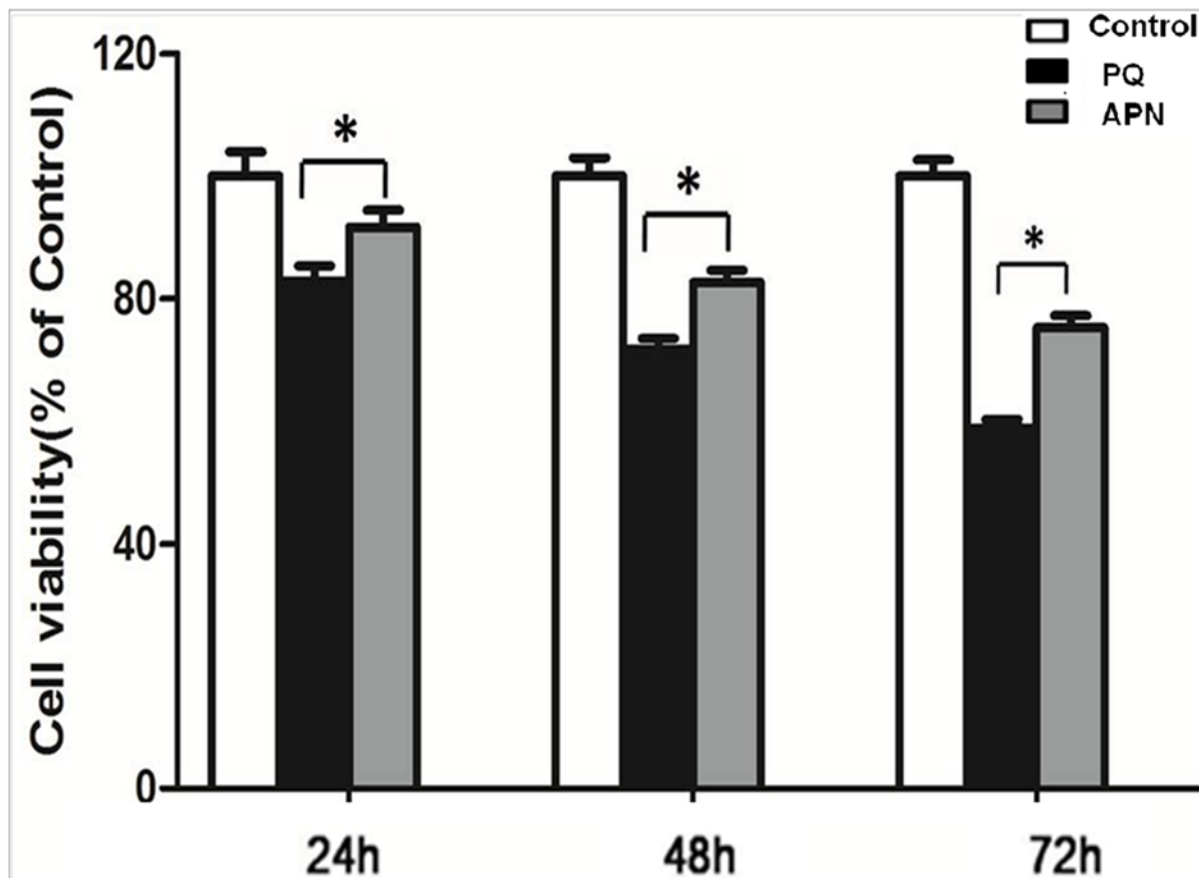
### APN attenuates PQ-induced cytotoxicity in human lung fibroblasts

Fibroblasts are integral in lung fibrosis development [17]. We employed the normal human lung fibroblast cell line WI-38, to determine the effect of APN upon PQ-induced cytotoxicity. First, we measured cellular viability via CCK-8. Incubation with PQ (0.3 mmol/L, 24 hours) decreased lung fibroblast viability in time dependent manner. Pretreatment with APN (5 µg/mL, 2 hours) significantly increased cellular survival rate ( $P<0.05$ , Fig 6).

Cellular apoptosis was assessed by Annexin V-FITC/PI staining and flow cytometry. Annexin V and PI negative cells (lower left quadrant) were considered nonapoptotic, viable cells. Dead cells are PI positive. Annexin V positive, but PI negative, cells are apoptotic. APN pretreatment (5 µg/mL, 2 hours) significantly decreased the apoptotic and dead population of lung fibroblasts subjected to PQ exposure (0.3 mmol/L, 24 hours) (Fig 7).

### APN decreases PQ-induced increased collagen type III expression in human lung fibroblasts

We specifically determined the effect of PQ on collagen type III expression, the main extracellular matrix component in pulmonary cells. Collagen type III deposition increases during acute lung injury and pulmonary fibrosis [18]. Employing Western blot and RT-PCR analysis, we



**Fig 6. Effect of APN on cell viability in control or PQ-incubated lung fibroblast.** PQ (0.3 mmol/L, 24 hours) decreased, whereas pretreated with APN (5 ug/mL, 2 hours) rescued, lung fibroblast viability at 24, 48 and 72 h after PQ incubation. Cell viability was measured via CCK-8 assay. Values represent the mean  $\pm$  standard error of the mean of three parallel measurements. \* $P < 0.05$ . APN, adiponectin; PQ, paraquat.

doi:10.1371/journal.pone.0125169.g006

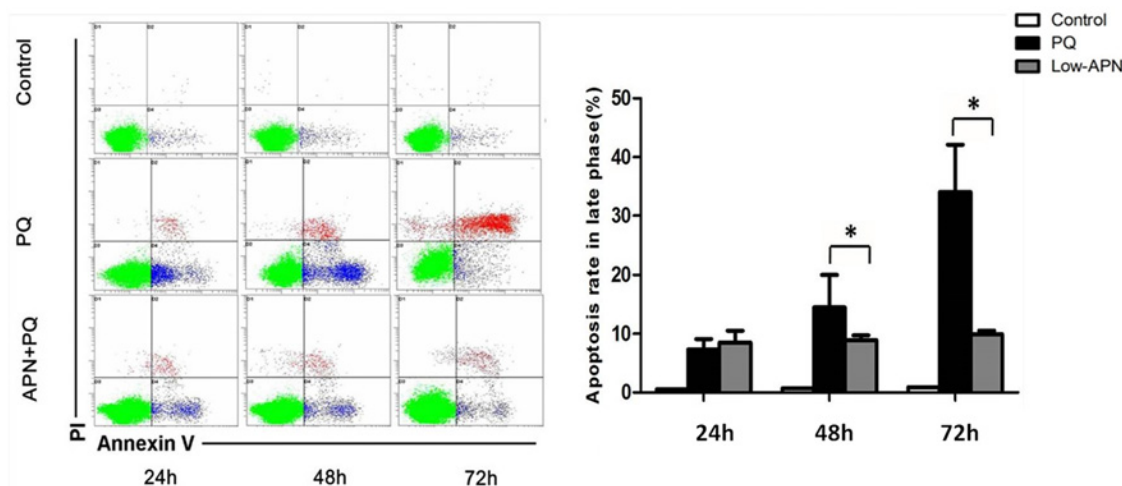
demonstrated APN pretreatment (5 ug/mL for 2 hours) markedly inhibited PQ—induced collagen type III expression in human lung fibroblasts ( $P < 0.05$ , Fig 8), suggesting PQ-induced ECM formation was significantly reversed by APN.

### APN receptor expression in lung fibroblasts WI-38

Previous studies indicate APN exerts anti-fibrotic effect by reducing inflammatory reaction or oxidative stress [8, 19]. We hypothesize APN influences PQ-mediated lung fibrosis directly via lung fibroblasts, a predominant effector cell in organ fibrosis. To test this, we determined whether purified populations of human lung fibroblasts WI-38 express the APN receptors (AdipoR1 and AdipoR2). Western blot and RT-PCR analysis demonstrate both AdipoR1 and AdipoR2 protein and mRNA are expressed in WI-38 lung fibroblasts. When cells were incubated with PQ for 24 hours, protein/mRNA expression of both AdipoR1 and AdipoR2 were up-regulated. APN pretreatment further enhanced AdipoR1, but not AdipoR2, up-regulation (Fig 9).

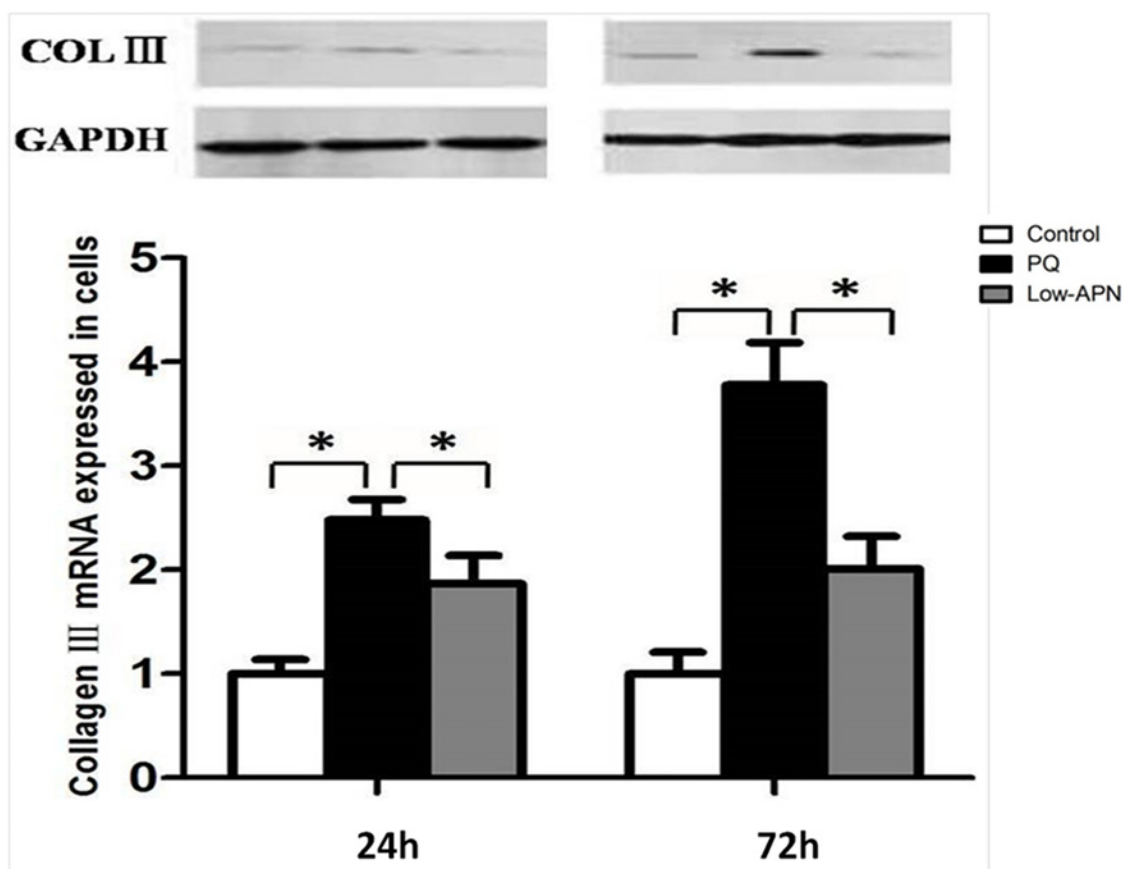
### AdipoR1-specific siRNA reverses the protective effect of APN against PQ-mediated fibrosis in lung fibroblasts WI-38

Experiment 3.6 suggests AdipoR1, not AdipoR2, may be involved in APN-mediated protective effect against PQ-mediated fibrotic effects. To determine the functionality of AdipoR1



**Fig 7. Effect of APN on cell apoptosis in control or PQ-incubated lung fibroblast.** PQ (0.3 mmol/L, 24 hours) augmented, whereas pretreated with APN (5 ug/mL, 2 hours) rescued, lung fibroblast apoptotic rate at 24, 48 and 72 h after PQ incubation. Cell apoptosis was assessed by Annexin V-FITC/PI staining and flow cytometry. Values represent the mean  $\pm$  standard error of the mean of three parallel measurements. \*P<0.05. APN, adiponectin; PQ, paraquat.

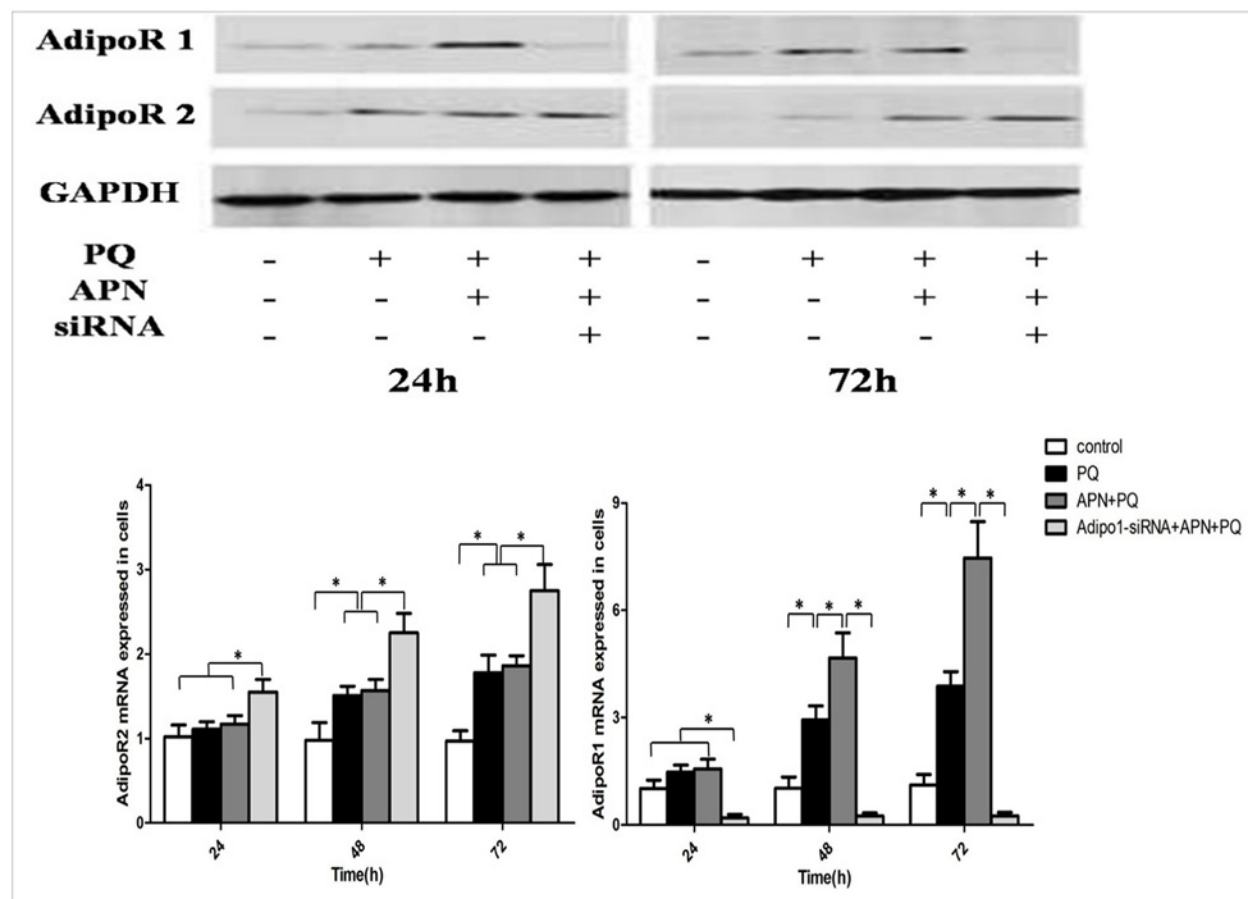
doi:10.1371/journal.pone.0125169.g007



**Fig 8. Effect of APN on collagen type III expression in control or PQ-incubated lung fibroblast.** PQ (0.3 mmol/L, 24 hours) increased, whereas pretreated with APN (5 ug/mL, 2 hours) rescued, collagen type III expression in lung fibroblasts at 24 and 72 h after PQ incubation. The collagen type III protein and mRNA expression were a determined via Western blot and RT-PCR, respectively. Values represent the mean  $\pm$  standard error of the mean of three parallel measurements. \* P<0.05. APN, adiponectin; PQ, paraquat.

doi:10.1371/journal.pone.0125169.g008





**Fig 9. APN receptor expression in lung fibroblast.** Both AdipoR1 and AdipoR2 expression were detected in WI-38 lung fibroblasts. PQ (0.3 mmol/L, 24 hours) treatments up-regulated protein and mRNA expression of both APN receptors in lung fibroblasts WI-38 at 24, 48 and 72 h after PQ incubation. APN (5 ug/mL, 2 hours) pretreatment further enhanced AdipoR1, but not AdipoR2, up-regulation. AdipoR1-specific siRNA successfully abrogated AdipoR1 expression. AdipoR1 and AdipoR2 expression were determined by Western blot and RT-PCR. Values represent the mean  $\pm$  standard error of the mean of four parallel measurements. \* $P < 0.05$ . APN, adiponectin; PQ, paraquat, AdipoR, APN receptor.

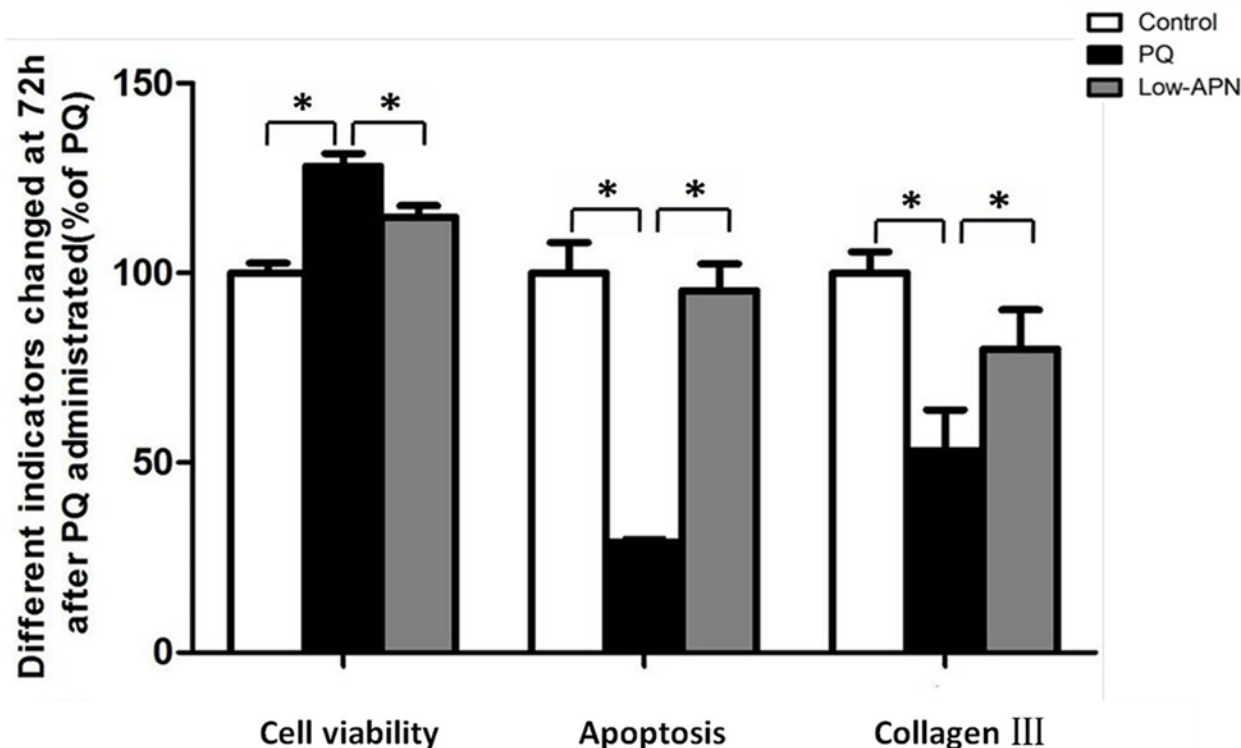
doi:10.1371/journal.pone.0125169.g009

expressed in lung fibroblasts, lung fibroblasts were transfected with AdipoR1-specific siRNA for 48 hours, and incubated with 5ug/mL APN for an additional 2 hours, and finally 0.3 mmol/L PQ for 24 hours. AdipoR1-specific siRNA successfully abrogated AdipoR1 expression (Fig 9). Knockdown of AdipoR1 significantly reversed the APN-mediated protective effect against PQ-mediated fibrotic effect (Fig 10). These data support the involvement of AdipoR1 expressed by human lung fibroblasts in APN-mediated protective effect against PQ-induced pulmonary fibrosis.

## Discussion

Pulmonary fibrosis is one of the most common and serious clinical sequela of PQ poisoning, responsible for high mortality [3, 4]. To date, no safe and effective treatments exist halting and/or reversing fibrotic pathophysiology. In the present study, we demonstrated APN, an adipocytokine with a wide range of biologic effects, exerts protective effect against PQ-induced pro-fibrogenic processes.

To discern the responsible underlying mechanisms for the observed phenomenon, we determined the expression pattern of fibrosis-related cytokines in mouse lung tissue. Of the relevant



**Fig 10. AdipoR1-specific siRNA reversed the protective effect of APN against PQ-mediated fibrosis in lung fibroblasts WI-38 at 72 h after PQ incubation.** Cell viability, apoptosis and collagen type III mRNA expression in lung fibroblasts were measured via CCK-8 assay, Annexin V-FITC/PI staining with flow cytometry, and RT-PCR, respectively. Values represent the mean  $\pm$  standard error of the mean of three parallel measurements. \* $P < 0.05$ . APN, adiponectin; PQ, paraquat, AdipoR, APN receptor.

doi:10.1371/journal.pone.0125169.g010

cytokines, TGF- $\beta$  is the most important pro-fibrotic cytokine mediating myofibroblast formation and ECM production [11, 12, 20]. As excessive fibroproliferation and ECM accumulation are critical determinants of fibrogenesis, TGF- $\beta$  inhibition has successfully reduced fibrosis development in different experimental models [21, 22]. CTGF has been implicated as both a downstream and cooperative mediator of TGF- $\beta$ , inducing fibroblasts to become myofibroblasts [13]. CTGF also directly or indirectly enhances the expression of its own inducer, TGF- $\beta$ , in positive feedback manner [23–25]. In our study, we demonstrated PQ increased expression of TGF- $\beta_1$  and CTGF, which was significantly reversed by APN treatment, suggesting the anti-fibrotic effect of APN is mediated partly via inhibition of TGF- $\beta_1$ /CTGF signaling.

It is well established that the myofibroblast is the ultimate effector cell in pulmonary fibrosis. As a differentiated fibroblast, myofibroblasts are the key source of ECM and various inflammatory cytokines, including TGF- $\beta_1$  [26], and are commonly identified by expression of  $\alpha$ -SMA. In the current study, APN treatment inhibited fibroblast differentiation into myofibroblasts.

The expression of MMPs and their physiologic inhibitors TIMPs are integral to fibrotic tissue development [27, 28]. While many MMPs are purportedly involved in pulmonary fibrosis, MMP-9 is one of the most relevant pro-fibrotic members of the family [14]. Increased MMP-9 is observed in clinical and experimental models of fibrosis [29]. TIMP-1, an inhibitor of MMP-9, is up-regulated during fibrosis [15, 16]. In the current study, marked increase of MMP-9 and TIMP-1 were observed in mice subjected to PQ exposure. Moreover, increased MMP-9 expression preceded increased TIMP-1 expression, the former peaking on day 3, and the latter peaking on day 7, a pattern that supports the notion an imbalance between matrix degradation and

its inhibition may contribute to PQ-induced pulmonary fibrosis. APN treatment results in decreased MMP-9 and TIMP-1 levels, and simultaneous peaking of both proteins on day 7, indicating correction of PQ-induced imbalanced MMP-9/TIMP-1 ratio.

In human lung fibroblasts WI-38 subjected to PQ in vitro, PQ decreased cellular viability, and increased apoptosis. APN significantly decreased PQ-induced cytotoxicity. PQ also markedly increased collagen type III synthesis (a major ECM component), an effect inhibited by APN in dose-dependent fashion.

To test our hypothesis APN influences pulmonary fibrosis directly via lung fibroblasts, we confirmed the presence of AdipoR1 and AdipoR2 (the two known transmembrane APN receptors) in human lung fibroblasts WI-38 via Western and RT-PCR. APN up-regulated AdipoR1, but not AdipoR2, expression in lung fibroblasts exposed to PQ. Additionally, siRNA-mediated knockdown of AdipoR1 abrogated APN's protective effects against PQ-mediated fibrosis. Together, these data support the functionality and possible therapeutic potential of AdipoR1 against fibrosis development post-PQ exposure.

The current study is important for several reasons. Firstly, our data provides the first evidence that exogenously administered globular isoform of APN mitigates the PQ-mediated pulmonary fibrogenic response. Secondly, our data supports the responsible mechanism underlying APN-mediated anti-fibrotic effect may involve 1) inhibition of TGF- $\beta$ <sub>1</sub>/CTGF signaling and 2) correction of the PQ-induced imbalance of MMP-9/TIMP-1 ratio, ultimately blocking the differentiation of fibroblasts into myofibroblasts. Thirdly, we demonstrate lung fibroblasts express functional AdipoR1, supporting the potential utilization of APN as an agent mitigating lung fibrosis. Our current works have some limitations. Considering the wide range of biologic effects of APN, there may be far more complex mechanisms than we had mentioned in this manuscript. For example, whether APN is involved in prevention of epithelial cells death, survival, transformation, or other important fibrosis related events, in context of PQ-induced fibrosis remain to be confirmed by further studies. In addition, mechanisms in PQ-induced lung injury could be different with that in other types of lung fibrosis, such as idiopathic pulmonary fibrosis.

In summary, we have demonstrated the pathogenicity of PQ upon pulmonary tissue in an animal model. In dose-dependent manner, APN mitigates PQ-mediated pulmonary fibrosis, via suppression of fibroblasts activation and differentiation. We present for the first time a novel therapeutic agent with exciting application for a clinical disease currently lacking efficacious therapy. Exploring the therapeutic and chemoprotective potentials of APN for treatment and prevention of pathologic pulmonary fibrosis in future toxicologic studies is warranted.

## Acknowledgments

We gratefully acknowledge Professor Xin-liang Ma of Thomas Jefferson University, Philadelphia, PA for his help and advice aiding successful completion of this study.

This work was supported by the National Natural Science Foundation of China (No. 30900493, 81471836) and the Science and Technology Department Foundation of Sichuan Province (No. 2013JY0011).

## Author Contributions

Conceived and designed the experiments: YC ZL ZZ. Performed the experiments: RY YH. Analyzed the data: WBL ZL. Contributed reagents/materials/analysis tools: ZZ ZL YC. Wrote the paper: WBL RY YH. Revised the manuscript as reviewer and editors required: WBL RY.

## References

1. Dinis-Oliveira RJ, Duarte JA, Sanchez-Navarro A, Remiao F, Bastos ML, Carvalho F. Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. *Crit Rev Toxicol*. 2008; 38: 13–71. PMID: [18161502](#)
2. Hoet PH, Lewis CP, Demedts M, Nemery B. Putrescine and paraquat uptake in human lung slices and isolated type II pneumocytes. *Biochem Pharmacol*. 1994; 48: 517–524. PMID: [8068038](#)
3. Yamashita M, Yamashita M, Ando Y. A long-term follow-up of lung function in survivors of paraquat poisoning. *Hum Exp Toxicol*. 2000; 19: 99–103. PMID: [10773838](#)
4. Suntres ZE. Role of antioxidants in paraquat toxicity. *Toxicology*. 2002; 180: 65–77. PMID: [12324200](#)
5. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends In Endocrinology And Metabolism*. 2002; 13: 84–89. PMID: [11854024](#)
6. Okamoto Y, Kihara S, Funahashi T, Matsuzawa Y, Libby P. Adiponectin: a key adipocytokine in metabolic syndrome. *Clin Sci (Lond)*. 2006; 110: 267–278. PMID: [16464169](#)
7. Robinson K, Prins J, Venkatesh B. Clinical review: adiponectin biology and its role in inflammation and critical illness. *Crit Care*. 2011; 15: 221. doi: [10.1186/cc10021](#) PMID: [21586104](#)
8. Kamada Y, Tamura S, Kiso S, Matsumoto H, Saji Y, Yoshida Y, et al. Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. *Gastroenterology*. 2003; 125: 1796–1807. PMID: [14724832](#)
9. Ohashi K, Iwatani H, Kihara S, Nakagawa Y, Komura N, Fujita K, et al. Exacerbation of albuminuria and renal fibrosis in subtotal renal ablation model of adiponectin-knockout mice. *Arterioscler Thromb Vasc Biol*. 2007; 27: 1910–1917. PMID: [17626903](#)
10. Fujita K, Maeda N, Sonoda M, Ohashi K, Hibuse T, Nishizawa H, et al. Adiponectin protects against angiotensin II-induced cardiac fibrosis through activation of PPAR- $\alpha$ . *Arteriosclerosis Thrombosis And Vascular Biology*. 2008; 28: 863–870. doi: [10.1161/ATVBAHA.107.156687](#) PMID: [18309113](#)
11. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol*. 1988; 41: 467–470. PMID: [3366935](#)
12. Bartram U, Speer CP. The role of transforming growth factor beta in lung development and disease. *Chest*. 2004; 125: 754–765. PMID: [14769761](#)
13. Meyer-Ter-Vehn T, Gebhardt S, Sebald W, Buttmann M, Grehn F, Schlunck G, et al. p38 inhibitors prevent TGF- $\beta$ -induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci*. 2006; 47: 1500–1509. PMID: [16565385](#)
14. Giannandrea M, Parks WC. Diverse functions of matrix metalloproteinases during fibrosis. *Dis Model Mech*. 2014; 7: 193–203. doi: [10.1242/dmm.012062](#) PMID: [24713275](#)
15. Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol*. 2003; 28: 12–24. PMID: [12495928](#)
16. Ruiz V, Ordonez RM, Berumen J, Ramirez R, Uhal B, Becerril C, et al. Unbalanced collagenases/TIMP-1 expression and epithelial apoptosis in experimental lung fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2003; 285: L1026–1036. PMID: [12882763](#)
17. Keeley EC, Mehrad B, Strieter RM. The role of fibrocytes in fibrotic diseases of the lungs and heart. *Fibrogenesis Tissue Repair*. 2011; 4: 2. doi: [10.1186/1755-1536-4-2](#) PMID: [21219601](#)
18. Lai CK, Wallace WD, Fishbein MC. Histopathology of pulmonary fibrotic disorders. *Seminars In Respiratory And Critical Care Medicine*. 2006; 27: 613–622. PMID: [17195138](#)
19. Krenning G, Moonen JRAJ, Harmsen MC. Pleiotropism of Adiponectin Inflammation, Neovascularization, and Fibrosis. *Circulation Research*. 2009; 104: 1029–1031. doi: [10.1161/CIRCRESAHA.109.198044](#) PMID: [19423859](#)
20. Luo KX. Ski and SnoN: negative regulators of TGF- $\beta$  signaling. *Current Opinion In Genetics & Development*. 2004; 14: 65–70.
21. Scotton CJ, Chambers RC. Molecular targets in pulmonary fibrosis—The myofibroblast in focus. *Chest*. 2007; 132: 1311–1321. PMID: [17934117](#)
22. Bronnum H, Eskildsen T, Andersen DC, Schneider M, Sheikh SP. IL-1 $\beta$  suppresses TGF- $\beta$ -mediated myofibroblast differentiation in cardiac fibroblasts. *Growth Factors*. 2013; 31: 81–89. doi: [10.3109/08977194.2013.787994](#) PMID: [23734837](#)
23. Wang Q, Usinger W, Nichols B, Gray J, Xu L, Seeley TW, et al. Cooperative interaction of CTGF and TGF- $\beta$  in animal models of fibrotic disease. *Fibrogenesis Tissue Repair*. 2011; 4: 4. doi: [10.1186/1755-1536-4-4](#) PMID: [21284856](#)

24. Brigstock DR. Strategies for blocking the fibrogenic actions of connective tissue growth factor (CCN2): From pharmacological inhibition in vitro to targeted siRNA therapy in vivo. *J Cell Commun Signal*. 2009; 3: 5–18. doi: [10.1007/s12079-009-0043-9](https://doi.org/10.1007/s12079-009-0043-9) PMID: [19294531](https://pubmed.ncbi.nlm.nih.gov/19294531/)
25. George J, Tsutsumi M. siRNA-mediated knockdown of connective tissue growth factor prevents N-nitrosodimethylamine-induced hepatic fibrosis in rats. *Gene Ther*. 2007; 14: 790–803. PMID: [17344905](https://pubmed.ncbi.nlm.nih.gov/17344905/)
26. Yokoi H, Mukoyama M, Nagae T, Mori K, Suganami T, Sawai K, et al. Reduction in connective tissue growth factor by antisense treatment ameliorates renal tubulointerstitial fibrosis. *J Am Soc Nephrol*. 2004; 15: 1430–1440. PMID: [15153554](https://pubmed.ncbi.nlm.nih.gov/15153554/)
27. Phan SH. The myofibroblast in pulmonary fibrosis. *Chest*. 2002; 122: 286S–289S. PMID: [12475801](https://pubmed.ncbi.nlm.nih.gov/12475801/)
28. Pardo A, Selman M. Matrix metalloproteases in aberrant fibrotic tissue remodeling. *Proc Am Thorac Soc*. 2006; 3: 383–388. PMID: [16738205](https://pubmed.ncbi.nlm.nih.gov/16738205/)
29. Lim DH, Cho JY, Miller M, McElwain K, McElwain S, Broide DH. Reduced peribronchial fibrosis in allergen-challenged MMP-9-deficient mice. *Am J Physiol Lung Cell Mol Physiol*. 2006; 291: L265–271. PMID: [16825657](https://pubmed.ncbi.nlm.nih.gov/16825657/)